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The major objective of our studies was to contribute to the understanding of cellular interactions that occur during the process of wound healing. The specific aim being to determine the role of cultured epidermal cells and growth factors produced by them in re-epithelization and formation of neodermis during would healing.

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The major objective of our studies was to contribute to the understanding of cellular interactions that occur during the process of wound healing. The specific aim being to determine the role of cultured epidermal cells and growth factors produced by them in re-epithelization and formation of neo-dermis during wound healing.

We have accomplished the following:

Using an experimental model system of surgically created partial and full thickness wounds we repeatedly documented the beneficial effects of autologous epidermal sheets on healing of such wounds. Moreover, we have shown that supernatant fluids of epidermal cells grown in tissue culture or cell extracts thereof influenced the healing of partial thickness wounds by increasing the rate of reepithelization and suppressing the scar tissue formation(Encl.1,2a,b). Based on these preliminary results we concluded that epidermal cells might be producing a factor(s) that regulates scar tissue formation. Although aware that complex interactions between different cell types within a healing wound may contribute to the outcome of wound repair, we chose to study the effects of epidermal cell extracts on fibroblasts. It was rationalized that fibroblast is the main cell type that contributes to scar tissue formation by excessive proliferation and production of different types of collagens. An in vitro system in which fibroblasts are embedded into collagen gels was developed by others and has been accepted as a model that mimics the process of wound contraction. Growth factors that have been shown to increase scar tissue formation in *vivo* (i.e. TGFß1) have been also shown to increase the rate of collagen contraction by fibroblasts in *vitro* . Epidermal cell supernatants and cell extracts inhibited this process profoundly, suggesting presence of inhibitor(s). We named this inhibitory activity associated with not yet identified molecule - Epidermal Cell Derived Factor (EDF). The collagen contraction inhibition procedure was then upscaled for handling large numbers of samples from experiments aimed at the biochemical characterization of EDF(s). Supernatant fluids of epidermal cell cultures and extracts of epidermal cells grown in tissue culture were tested as possible sources for purification of the factor(s), but substituted later by epidermal cells obtained from animals following a superficial wounding. Cell extracts prepared from such regenerating epidermal cells have been found to be a relatively rich source of EDF activity compared to other tissues tested.

As detailed in the enclosed publication(Encl.3), EDF activity in the epidermal cell extracts was found to be destroyed in acid environment (pH3), but stable in alkaline (pH10), stable at 56° C but inactivated by 100° C. It was found to be stable at -20° C for many months and at 4° C for two weeks.

When separated by gel filtration chromatography the activity was found in three areas corresponding in molecular weight to 30, 5 and 1 kD. Of different ion exchange columns only Q Sepharose was repeatedly shown to bind EDF(s)

at pH 8 with high affinity. Elution was achieved with 0.5 M NaCl. Further purification of the factor was by FPLC mono - Q exchanger and Reversed phase chromatography. Following the first purification steps the biological activity in *vivo* was also retained (Encl3)
Preliminary experiments using small columns and relatively small amounts of starting material were encouraging, biological activity following the above purification steps was always detectable. The quantities of materials so obtained were, however, insufficient for further biochemical characterization and molecular identification of the EDF. Thus major effort was made to collect large amount of starting material, scale up the purification procedure. Subsequently, material from 3 large and 4 small gel filtration columns (about 490 ml of starting material) corresponding to 30 kD EDF was pooled and applied to a Q Sepharose, followed by Mono Q column and reversed phase chromatography. Following this protocol biological activity was detected in two consecutive and one separate fraction eluted from reversed phase chromatography column. Biological activity of the single fraction coincided with a small protein peak. Most of this material was therefore subjected to gel electrophoresis to assure separation of proteins. However, following this separation the material in the gel was not detectable by either Comassie blue or Silver stains. Subsequently, the remaining material (45 microliters) was subjected to preparative electrophoresis. There was neither detectable O.D. (at 218 nm) nor biological activity in fractions collected. Fractions were tested in a collagen contraction micro assay. The results of this series of experiments were disappointing and led to the following conclusions: 1) In order to isolate by the outlined purification scheme sufficient amount of material for the identification of the inhibitory molecule it will be necessary to prepare 10-100 times more starting material and 2) The inhibitory activity detected in epidermal cell extracts may result from concerted action of more than one factor; their separation by chromatography resulting in a gradual loss of activity. The possibility to prepare 10 or 100 times more starting material was considered but found objectionable unless good evidence can be obtained that EDF activity is associated with a single previously unidentified factor. Therefore a large battery of known factors that could be present in an epidermal cell extract were examined at different concentrations. We found Interleukin 1 (α and β), Retinoic Acid, Prostaglandin E-2 (PGE-2) and Atrial Natriuretic Polypeptide, known for its vasodilating effects, all effective in inhibiting collagen contraction by fibroblasts. Epidermal Growth Factor, Platelet Derived Growth Factor and Interleukin 2 showed no significant effect; Nerve Growth Factor and Transforming Growth Factor β1 actually increased fibroblast induced contraction. Of the growth factors shown to inhibit collagen contraction Il-1 was the most potent candidate. Reexamination and evaluation of the epidermal extracts and fractions obtained from them has revealed presence of almost 2000 pg/ml of IL-1 in the starting material and increased amounts in the fractions that were used for further purification steps.

Immunoprecipitation and Western blot analysis using specific antibodies detecting pig IL-1 α or β, documented presence of IL-1 α predominantly. However a small amount of IL-1β was also detectable. This finding suggested a possible role for keratinocyte produced IL-1 in the initiation of the inflammatory phase of the wound healing process. Based on the known growth factors transcriptional cascade associated with an inflammatory response, IL-1 was an unlikely candidate to be involved in the inhibition of scar tissue formation in *vivo*. As to the in *vitro* inhibitory effect of IL-1 on collagen contraction, it may be contributed to PGE-2. Prostaglandin E-2 was previously shown to inhibit collagen contraction by fibroblasts. Conversely, IL-1 has been shown to induce synovial cells to produce PGE-2. Provided IL-1 has the same effect on the fibroblasts used in our assays, the increased PGE-2 production by these cells may be responsible for the inhibition of collagen contraction seen by IL-1, possibly due to increased collagenase production. Thus, as documented by our studies, isolation of a factor that inhibits scar tissue formation in *vivo* does not seem feasible when aided by the collagen contraction inhibition assay

Despite these discouraging results, acutely cognizant of a need for substances that would be negative regulators of scar tissue formation we turned to fetal tissues. Awareness existed that fetal wounding in certain animals, including pigs, result in skin regeneration, rather than its repair by excessive scar tissue formation. Thus we examined the possibility of using epidermis from pig fetuses and also tested amniotic membranes associated with them. The separation of epidermal cells from pig fetal tissues has proven too difficult for large scale studies, thus their use was impractical. In addition IL-1 was also detected in the fetal skin in high concentrations, excluding the possibility of using collagen contraction inhibition assay for purification of such a factor. The possibility to isolate EDF from fetal skin was abandoned. Amniotic membranes, associated with pig fetuses were also tested for IL-1 and collagen contraction inhibitory activity. Since they had no IL-1 activity and showed relatively good collagen contraction inhibitory activity a batch of amniotic membranes derived from 100 pig fetuses was collected at full gestation and subjected to purification. There was no good separation of the biological activity from this starting material when subjected to a purification procedure similar to that used previously for epidermal cell extracts.

The studies presented here document the need for a more effective and predictive in vitro assay system for evaluation of scar tissue formation. Since TGFβ3 has been recently suggested to be a negative regulator of scar tissue formation in *vivo* and it is produced by epidermal cells, the inhibition of scar tissue formation we observed in *vivo* may have been due in part to TGFβ 3 activity. Innovative ideas and new approaches are awaited to answer the question about the possible role of epidermis and its factors in the negative regulation of scar tissue formation.

Studies aimed at the understanding of the possible role for growth factors in allogeneic epidermal cell graft rejection were detailed in the midterm report.

(see enclosure 4). Suggestive evidence for auto induction of gamma interferon by allogeneic keratinocytes, but not fibroblasts, leading to the expression of class II antigens was presented. It was speculated that auto induction of Ia antigens may contribute to recognition of allogeneic epidermal cells by T - lymphocytes and graft rejection.

II. STUDIES AIMED AT THE UNDERSTANDING OF ALLOGENEIC EPIDERMAL CELL GRAFT REJECTION

Epidermis, the outermost layer of skin, is composed of stratified squamous epithelium. Its function is to provide a physiological barrier and protection of the body. Because of its functional and cosmetic importance means for epidermal cell replacement, when lost by injury, have long been sought.

As skin has proven to be one of the organs most difficult to transplant allogeneically, findings that epidermal cells when grown in tissue culture can be freed of Langerhans cells, led to the belief that new approaches to allogeneic epidermal cell transplantation will become available. However, based on the work of many investigators, it became obvious that even in the absence of Langerhans cells, the antigen presenting cells of the epidermis, allogeneic epidermal keratinocytes were always rejected. Thus, our goal was to understand the mechanism of allogeneic epidemal cell graft rejection.

Induction of Class II Major Histocompatability Antigens (MHC) on Keratinocytes by Allogeneic Dermis

It was reported previously (4,5,6), that in the skin transplanted allogeneically, early in the process of rejection, epidermal cells were induced to expression of class II major histocompatibility antigens, Ia antigens. These antigens, found to be constitutively expressed on Langerhans cells of the epidermis, and are known to be necessary for antigen presentation to T-lymphocytes, cells responsible for graft rejection. It was presumed that Ia induction on epidermal cells was secondary, caused by gamma interferon, produced by lymphocytes, present in rejecting grafts. This immune interferon has been shown by many, to be able to induce Ia expression not only on keratinocytes, but also on fibroblasts. To investigate whether the mechanism of class II induction on keratinocytes is due to exogeneous gamma interferon, we etablished an in vitro system. The system was described in detail in the summary report of our previous contract.

A) <u>In Vitro Ia Induction Assay</u>

Briefly, human or pig keratinocytes were grown in tissue culture until multilayer

epidermal structures were formed. They were removed from the tissue culture vessels as sheets, using the enzyme Dispase. A split thickness graft of reticular dermis was obtained from pigs, using a Brown dermatome. Following thorough wash of the dermis, epidermal graft was "transplanted" on the dermis and the composite graft placed onto a stainless steel grid in a Petri dish filled with tissue culture medium.

In experiments with human keratinocytes grown in vitro for 3 weeks and placed on pig dermis, induction on Ia antigen was observed in the following fashion: on **Day 3** - all cells were Ia^- (negative); on **Day 5** - 20% of keratinocytes were Ia^+ (positive); subsequent increase to 40% was found on **Day 7** and **Day 9. Day 11** - the number of positive cells reached 90%. This gradual increase was followed by a decrease of Ia^+ cells on **Days 13** and **18** to 15%. Human fibroblasts exposed to the dermis of the same donor as were keratinocytes in the above experiments, were not induced to Ia expression. Negative results obtained with fibroblasts suggested that the induction of class II histocompatibility antigens seen with keratinocytes was not due to the presence of exogeneous interferons in the dermis, but that if interferons are the cause they may be autocrine in nature. To confirm an already well accepted finding that gamma-interferon can be an inducer of Ia expression in fibroblasts, and that this finding applies to the fibroblasts used in the above studies, we tested recombinant gamma-interferon on the above fibroblasts and showed induction of Ia antigens with 100 U/ml or gamma-interferon. Thus, we provided further evidence that induction of class II major histocompatibility antigens on epidermal keratinocytes, when exposed to allogeneic dermis was not due to exogeneous gamma interferon.

B) Neutralization Assay

To confirm and clarify our preliminary evidence that expression of Ia antigens on keratinocytes may be due to induction of an autocrine gamma-interferon, neutralization assays with anti-gamma interferon were performed. The assay system used was modified to use less media, therefore to require less of costly antibodies. Smaller dishes were custom made for us by the mechanical shops of SKI. These dishes needed only 10 ml media/each, compared to 50 ml used previously. The experiments were set as previously described using fresh pig dermis and human keratinocytes

grown in tissue culture. Anti human gamma-interferon antibodies at 1 U/ml were added at the time of co-cultivation and every second day the medium was exchanged for the same. As shown on the enclosed graph (Fig. 7), 90% induction of Ia antigen was observed on day 7 in the control samples. In contrast, in the presence of antibodies to gamma interferon a significant suppression of Ia induction was found (15% positive cells). In another assay (not shown) a complete suppression of Ia-antigen was seen in the presence of anti-gamma interferon antibodies. In this case, however, controls (in the absence of antibodies) showed 25% maximal induction only.

C) Hybridization In Situ

In Addition to the above experiments, we also succeeded in obtaining good quality in situ hybridization data. They represent repeated, previously described experiments using a gamma interferon probe to localize messenger RNA in the epidermal cells cultured on the pig dermis. The data are now sufficient to support our findings that allogeneic or xenogeneic dermis can induce keratinocytes to gamma-interferon production and expression of class II antigens. Manuscript for publication is being prepared.

D) Induction of Class II MHC Antigens on Keratinocytes by "Treated" Dermis

It was reported previously (7) that frozen dermal allografts supported the engraftment and expansion of autologous epidermis. Naturally, it became of interest to examine whether frozen dermis retains its capacity to induce Ia on cultured keratinocytes. We therefore froze (pig) dermis, using the protocol previously described, and used it in in vitro "transplantation" experiments. Frozen dermis induced Ia expression in keratinocytes after 14-16 days of co-cultivation in vitro. The induction was however only 5% on days 14 and 16 and 10% on day 20, compared to the control, non-frozen dermis in which 40-90% of the epidermal cells were induced to express Ia antigens in 7-11 days. Repeated experiments confirmed our findings. In all instances we found that freezing significantly suppressed the dermal activity responsible for Ia induction in keratinocytes. However, repeated freeze - thawing (3X) did not have additional effects. For example: Controls were induced on day 5, reached maximum

on day 9 (60% positive cells) and declined gradually to day 20. In contrast, 1x frozen-defrosted dermis induced expression of Ia only after 12 days of cocultivation, and only in less than 10% of the cells. Three (3) times frozen dermis induced Ia expression after 14 days also on less than 10% of the cells tested. To eliminate the possibility that DMSO, used in the freezing procedure, might have interfered with the induction, a control experiment for the effect of DMSO was performed. It was found that when dermis was exposed to the same concentration of DMSO as used for freezing, subsequently washed but not frozen, induction of Ia by keratinocytes was not affected.

We also examined the effects of prolonged exposure of dermis to proteolytic enzymes such as trypsin (at 0.25%, 12 hrs at 4°C, concentration known not to be harmful to cells) on the Ia induction. We found that trypsinized dermis acted similarly as frozen dermis; Ia induction occurred later than in the controls (on day 12), but the numbers of positive cells were slightly higher than seen with frozen dermis (20-30%). A combination of trypsinization and freezing, however, completely abolished the activity. These findings may be important for possible clinical use of dermis in full thickness (third degree) burn wounds.

E) Induction of Class II MHC in "Conditioned" Epidermal Cells by Allogeneic Dermis

In addition we performed experiments using pig epidermal cells, "conditioned" with allogeneic dermal extracts, while grown in tissue culture and control cells "not conditioned". Both were subsequently "transplanted" in vitro onto allogeneic dermis from the same donor. These experiments were to assess whether "treatment" of cells with dermal extracts inhibits or delays Ia-induction by keratinocytes. Limited number of experiments of this nature were done, suggesting that Ia induction is delayed but not inhibited when epidermal cells are pre-treated. Thus, treatment of dermis appears to be a more effective way to influence Ia induction in keratinocytes than "treatment" of epidermis.

F) Induction of Ia Antigens on Human Keratinocytes by Tumor Necrosis Factor (TNF) or Combination of TNF and Interferons

We investigated whether TNF, known for its capacity to induce class II antigens on

different types of cells including endothelial cells, can alone or in combination with small doses of gamma-interferon cause induction of Ia on keratinocytes. TNF at 50, 500,and 5,000 U/ml; gamma-interferon at 1, 10, 100, and 200 U/ml; and alpha-interferon at 100, 200, and 1,000 U/ml was tested on human keratinocyte cultures. In addition, a combination of TNF (5,000 U/ml) and gamma-interferon (100 U/ml) as well as TNF (5,000 U/ml) with gamma-interferon (200 U/ml) were tested for their capacity to induce Ia in keratinocytes. Gamma-interferon at 100 and 200 U/ml induced Ia in 75% of keratinocytes. TNF at 5,000 U also appeared to induce Ia in keratinocytes. Alpha-interferon gave negative results. The finding that TNF might induce Ia in keratinocytes might be very interesting, but must be repeated. We also plan to test IL_6 at the same time. These experiments should provide a solid baseline study of inducibility of Ia antigens on human keratinocytes and lead to experiments deciphering the importance of individual factors in Ia expression and may be allograft rejection.

Clinical Examination of Autologous Frozen Dermis in Full Thickness Wounds

To evaluate the potential for clinical use of frozen dermis or frozen dermis covered by autologous epidermal cells grown in <u>vitro</u> we prepared a series of full thickness wound beds and transplanted: 1) dermis that was frozen-defrosted 1x; 2) dermis frozen-defrosted 3x; 3) frozen-thawed dermis co-cultured with autologous epidermal cell. In addition: 4) we covered one wound with split thickness fresh autograft and 5) with sheets of autologous epidermal cells grown in tissue culture and 6) a control wound was covered with petrolatum gauze. Each wound was approximately 25 cm^2. All grafts in this experiment were autologous. Clinical examination on day 6 suggested take of dermal autografts (both 1x and 3x frozen) but there was no epidermal coverage. On the dermal frozen graft that was covered by epidermal cells in tissue culture there was a thin epidermal coverage. Split thickness autograft, as expected, was completely healed. Tissue grown epidermal autograft provided a thin epidermal coverage. The control wound was completely open. On day 9 and 13 - wounds covered by epidermal sheets were completely healed, they felt soft and pliable. They resembled those of split thickness skin. Wounds covered by 1x and 3x frozen-thawed dermis looked identical. There was no wound contraction. On day 9 they were partly covered by epidermis.

On day 13 there was full epidermal coverage. Wounds covered by in vitro co-cultured epidermis with dermis were not confluently covered by epithelium on day 9, but complete coverage was noted on day 13. Interestingly, some hair growth occurred in this wound. Controls on day 9 and 13 were contracted, but the remaining area following contraction was still denuded.

Preliminary histological examination of biopsies taken on day 6 and 13 gave interesting insight into the process of wound healing under these different conditions.

Wound beds covered by epidermal sheets grown in tissue culture were fully covered by a thick epithelium on day 6. The hyperplasia observed in these biopsies regressed by day 13. A thick layer of granulation tissue filled the wound.

Significant difference was between the dermal grafts that were covered by epidermal cells and plain dermal grafts. There was much less granulation tissue on the interface between the dermal grafts and fat (the grafts were placed on fat) when grafts with epidermal cells were used, than with dermal grafts alone. In addition, the fibroblast infiltration of the dermis was substantially less in dermis-epidermal grafts. This result is not surprising, since we have originally found that epidermis produces a factor (EDF) that suppresses fibroblast migration/proliferation in the wounds.

It is natural that we are now planning to extend these experiments to the use of allogeneic dermis. We plan to use frozen dermis as well as frozen-trypsinized dermis for further studies.

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Enclosure 2a

Clinical and Experimental Approaches to Dermal and Epidermal Repair: Normal and Chronic Wounds, pages 375–389

EPIDERMAL CELL DERIVED FACTOR (EDF): PARTIAL PURIFICATION AND CHARACTERIZATION

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INTRODUCTION

Since the initial discovery of epidermal growth factor (EGF) and nerve growth factor (NGF) (Levi-Montalcini, Cohen, 1960; Cohen, 1962), large numbers of polypeptide growth factors have been isolated from different tissues (Burgess, 1988). They were shown to operate according to the same principles governing classic hormones and their receptors, and act in an autocrine or paracrine fashion. A variety of growth factors and lymphokines have also been shown to be produced by epidermal cells (Milestone and Edelson, 1988). Of these EGF, transforming growth factor-alpha (TGF-alpha), basic fibroblast growth factor (b-FGF) and interleukin-1-alpha (IL-1-alpha) have been implicated in the stimulation of epidermal cell regeneration. In ad-dition, potent inhibitors of epidermal cell proliferation (chalones) have been previously i-dentified in epidermal cells (Reichelt et al, 1987). The effects of most known and well char-acterized growth factors on fibroblasts have been shown to be stimulatory *in vivo*, resulting in an increase in fibroblast cell numbers and generation of granulation tissue.

We have shown previously, that epidermal cell extracts and supernatant fluids of cultured epidermal cells stimulated keratinocytes but inhibited fibroblasts. This biological activity was ascribed to an epidermal cell derived factor(s)

(EDF) (Eisinger et al, 1988a, 1988b). The *in vitro* effect of EDF on epidermal cells resulted in an increased number of rapidly proliferating colonies composed mainly of basal keratinocytes. In fibroblast cultures EDF inhibited the ability of fibroblasts to cause contraction of collagen sponges. Epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), platelet derived growth factor (PDGF), transforming growth factor ß (TGF-ß), nerve growth factor (NGF), and extracts of WI-38 cells (human embryonic lung fibroblasts) did not have this inhibitory activity. Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes from remnants of glands, hair follicles and wound edges. The restoration of complete epidermal coverage occurred twice as rapidly as that of the control wounds. In addition, regenerating dermis in the EDF treated wounds contained 1/5 to 1/15 as many cells as cells treated with EGF, urogastrone, TGF-ß or phosphate buffered saline.

These findings implied a possible role for EDF(s) in both positive and negative feedback mechanisms and the maintenance of homeostasis in the epidermis and dermis. In view of their importance for understanding the normal mechanism of wound healing and potential for controlling abnormal or clinically undesirable manifestations, i.e. scar formation, we attempted to biochemically purify and characterize the EDF molecules. This paper describes partial purification and characterization of EDF(s) derived from pig skin.

MATERIAL AND METHODS

Preparation of Epidermal Cell Extracts

Pig epidermal cells were grown in vitro as previously described (Eisinger, 1985). Confluent cultures (grown for 3-6 weeks) were washed twice with Dulbecco's phosphate-buffered saline (PBS), removed by scraping with a rubber policeman and pelleted at 180 x g for 10 min. In addition pig

skin, wounded 7 days prior to collection by superficial shaving with a Dermatome, was used as source of epidermal cells. Skin obtained from donor pigs was washed extensively in antibiotic mixture (penicillin, streptomycin, Fungizone), cut into strips and enzymatic separation of epidermis from dermis was achieved in a 0.25% trypsin solution (DIFCO 1:250) at 37°C for 2 hours or at 4°C overnight. Following a thorough wash in PBS the epidermis was mechanically separated from the dermis using watchmaker forceps. Epidermal cells were pelleted at 180xg. The pellets were resuspended in an equal volume of PBS, sonicated twice for 15 seconds, and diluted 1:2.5 in PBS. The suspension was clarified by two consecutive ultracentrifugation steps at 16,000xg for 20 min. and 150,000xg for 45 min. The resulting clarified epidermal cell extract, considered 1:2.5 diluted starting material was divided into aliquots and frozen at -70°C.

Measurement of Fibroblast Contractility In Vitro

Human foreskin fibroblasts, HFSF-132 (kindly provided by P. Ehrlich) were grown in tissue culture in minimal essential medium with Earle's salts, containing nonessential amino acids, 2 mM L-glutamine, antibiotics and 7.5% fetal bovine serum (cMEM). Cells passaged 16-27 times were used for the experiments.

Three dimensional collagen gels were prepared by minor modification of previously described methods (Montesano, 1988; Bell et al, 1979; Buttle et al, 1983). Type I collagen was extracted by stirring adult rat tail tendons for 16 hrs at 4°C in a sterile 10 mM hydrochloric acid solution (300 ml for 1.5g of tendon). The resulting solution was centrifuged at 25,000xg for 50 min. at 4°C. The supernatant was than precipitated with sodium chloride at 10% final concentration. Mixing at 4°C for 6 hrs was followed by centrifugation at 30,000 xg for 50 min. at 4°C. The pelleted precipitate was re-dissolved in 2.5mM HCl (~ 100

ml) and stirred at 4°C for 24 hrs. Dialysis tubing (6-8,000 mwco) was used to extensively dialyze the material against 2.5 mM HCl.

For incorporation into collagen gels, cells were harvested from confluent cultures using 0.05% trypsin and 0.02% EDTA counted and adjusted to a concentration of 1 x 10^6 cells/ml in cMEM. They were placed on ice together with other ingredients. Each 35 mm plastic petri dish (Falcon, 1008) was filled with 0.6 ml collagen gel solution, 0.6 ml 2x MEM, 0.15 ml FCS, 0.05 ml MEM and 1x10^5 cells in 0.1 ml. They were allowed to gel for approximately 10 min at room temperature and the test material diluted in cMEM (total volume 0.5 ml) was layered on top of the gel. Twenty-four hrs later the gels were de-tached from the walls by gently tilting and rotating the dish and freeing the edges with a size 10 blade.

Gel contraction was quantified at 48-72 hrs after application of the test materials. The dishes were placed on a metric scale graph paper and the major and minor axes of the collagen gels were measured. The area of the gels was calculated and expressed as a percentage of the initial area. Inhibition of collagen contraction was calculated from the ratio of percent contraction of triplicate samples containing factor and control samples in the absence of factor. For the final evaluation values obtained for 100% contraction of control samples represented 0% inhibition of contraction.

For comparative studies of different EDF preparations, or fractions obtained in the process of purification, their collagen contraction inhibition activity was compared to an EDF standard. The standard was defined as 50% contraction inhibiting activity of an epidermal cell extract shown to be active in the assay to a final dilution of 1/3200. Inhibition of contraction by a tested sample which was equal to or less than 25% of the standard was considered negative.

Gel Filtration and Ion Exchange Chromatography

Concentrated starting material, (pig epidermal cell extract), (10 ml) was applied to a Sephacryl SF-300 (Pharmacia) column (2.5x95 cm), equilibrated in 20 mM $NaPO_4$, 0.15M NaCl, pH 8.0 and 5 ml fractions were collected at a flow rate of 4 ml/hr. The absorbance was measured at 280 nm. The column was calibrated using Blue dextran (2,000,000) Aldolase (158,000) Bovine serum albumin (68,000) Chymotrypsinogen A (25,000) and Phenol red (376). Two hundred microliter aliquots of 3 consecutive fractions were pooled and tested for fibroblast inhibitory activity in collagen gels. Fractions which test-ed positive, were pooled and diluted with 20 mM TRIS, pH9.0 to a 0.03M final concentration of sodium chloride. The material was loaded onto a Q sepharose (Pharmacia) column (1 x 5 cm), equilibrated in 20mM TRIS, 0.03M NaCl at pH 9.0. The column was washed with the same buffer, and eluted with a 0.1-0.7 M NaCl gradient. Aliquots of each 2 ml fraction, adjusted to 0.15 M salt concentration, were tested. Biologically active fractions were pooled again, clarified by centrifugation at 100,000xg for 1 hr, diluted with TRIS buffer to 0.03M final salt concentration and applied to FPLC-Mono Q column (Pharmacia) equilibrated with 0.05MNaCl in 20mM TRIS, pH8.0. Following application of the test material the column was washed with 0.05 M NaCl in TRIS buffer, and eluted with a multi-linear gradient of 0.05-2 M NaCl in 20mM TRIS, pH8.0. Aliquots from one ml fractions were tested for biological activity.

Assay of EDF Activity in Vivo

Domestic outbred swine were anesthetized with ketamine hydrochloride, anesthesia was maintained by a mixture of halothane, nitrous oxide, and oxygen. The operation site was shaved and cleansed with betadine and 70% (vol/vol) ethyl alcohol. Wounds 0.040 inch (1mm) deep were created on the sides of the thorax with a Brown dermatome. Nonadhesive dressing (Release, Johnson&Johnson) was cut to fit the size of the

wound, soaked in the materials to be tested, and applied to the wound bed. Control wounds were treated with Release soaked in PBS. The dressing was covered with multiple layers of gauze held in place by silk ligatures and protected by an Elastoplast bandage. After surgery the pigs received analgesics such as Tylenol to alleviate discomfort. The wounds were observed at 2 to 4 day intervals and 3 mm punch biopsy specimens were taken from the center of the wound. After 5 days the wounds were redressed with Release with saline solution only. By 12-14 days after surgery wounds were usually left uncovered.

Histology

The 3 mm punch biopsy specimens were fixed in Bouin's solution overnight and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and evaluated by light microscopy.

RESULTS

Activity of EDF(s) as measured by inhibition of fibroblast contraction of collagen gels

Collagen contraction by fibroblasts have been shown to be i)proportional to the number of cells embedded in the gel and ii) inversely related to the collagen concentration (Bell et al, 1979). We used a low concentration of collagen (1.3 mg/ml), and moderate number of fibroblasts (1×10^5/2 ml of gel mixture). Under these conditions fibroblasts produced a slow and moderate contraction of collagen gels and EDF inhibited contraction of collagen. The % inhibition of collagen contraction was dependent on the amount of EDF applied to the assay. A linear concentration dose dependent response was however, not achieved. Consequently the assay was considered semiquantitative only.

Aided by the EDF standard preparation, comparative studies of different cell extracts

were made. It was found that cell extracts prepared from cells grown in tissue culture and those actively growing *in vivo* after superficial wounding had similar collagen contraction inhibiting activity. On the other hand, non-wounded skin derived epidermal cell extracts had approximately 20 times less activity, suggesting a functional role for EDF(s) in the wound healing process. Since harvesting skin from pigs was much more economical than growing cells in culture, we used donor pigs as a source of starting material.

Biochemical Characteristics of EDF

Table 1

Treatment	Stability
pH 10.0	+
pH 2.0, 4.0	-
100°C - 10 min.	-
60°C - 30 min.	+
4°C - 2 weeks	+
-80°C - 1 month	+
-20°C - 1 month	+
Freeze-Thaw (6x)	+
Room Temp. - 2 weeks	+
Lyophilization	+
Acid Ethanol	-
Affinity Chromatography	**Binding**
Con A	-
Heparin	-
Hydroxylapatite	+/-
Blue Sepharose	-
Phenyl Sepharose	-
Lysine Sepharose	-
Octyl Sepharose	-
Remazol Blue Agarose	-
Dextran Sulfate Agarose	-
Ion Exchangers	**Binding**
CM Sephadex pH 6.0	-
DEAE Sephacel pH 8.0	+/-
Q Sepharose pH 8.0	+

Table 1 lists different treatments of epidermal cell extracts and their effects on biological activity of EDF(s) as detected by the collagen contraction assay. EDF(s) have been found to be stable at alkaline pH (pH 10) without any loss of the biological activity. In contrast, all EDF activity was lost when subjected to pH 4.0. Decline in the activity was found even at pH 5.5. EDF(s) are therefore acid sensitive, a property which interfered with the use of many

purification procedures that require low pH. When treated at 60°C for 30 min activity was retained, but titration of these material revealed an approximate 50% loss. All activity was lost by boiling for 10 min. The biological activity was retained at 4°C and room temperature for 2 weeks. Freeze thawing (6x) and storage at -80°C did not result in significant losses of the activity. When lyophilized all the activity was recovered following resuspension to the original volume in distilled water. To evaluate whether EDF activity is associated with a lipid containing molecule chloroform methanol and acetone extraction methods were applied. Repeated experiments have shown the material to be unextractable, suggesting it not to be lipid containing molecules. Attempts to bind EDF(s) to affinity chromatography columns have proven unsuccessful. From ion exchange chromatographies, Q sepharose was found to bind the activity at pH 8.0 and even better at pH 9.0.

Biological Activity of EDF(s) Purified by Gel Filtration and Ion Exchange Chromatography

Separation of EDF(s) based on their molecular weight was done by a Sephacryl SF-300 column. Application of 10 ml of starting material, resulted in the separation of 3 distinct peaks displaying biological activity (data not shown). Activity, eluted from SF-300 column prior to chymotrypsinogen (a molecular weight marker), was estimated to be approximately 30 kd and designated as higher molecular weight EDF (EDF-H). The activity detected at the tail end of the column was estimated to be approximately 1-2kd and designated low molecular weight EDF (EDF-L). An additional peak of biological activity was detected and estimated to be approximately 5 kd. EDF-L and EDF-H were futher purified and investigated. Fractions collected from SF-300 column and shown to have gel contraction inhibiting activity were pooled separately, based on their m.w., and applied to Q-Sepharose.

As shown in Fig 1A the elution of the biologically active EDF-L in a step-wise salt

gradient was achieved at 0.5 M NaCl concentration.

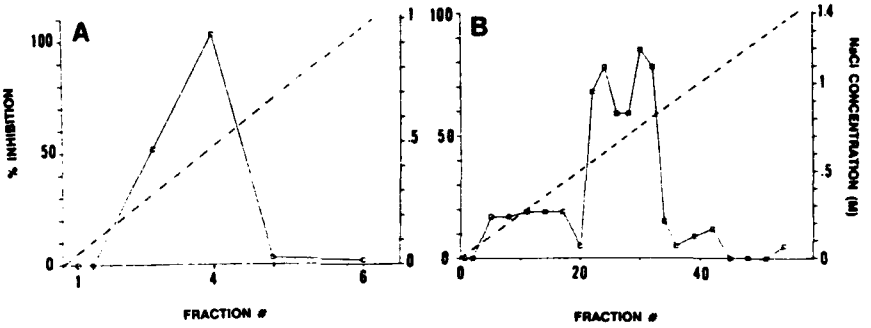

Fig. 1. Elution profile of EDF from ion exchange columns. **(A)** Q-Sepharose **(B)** FPLC Mono Q. The biological activity was detected by collagen contraction assay described in the text.

To concentrate the material and to purify it further, the pooled fractions were applied to an FPLC mono Q column (see Fig. 1B) and eluted with a linear gradient of 0.05-1.4 M NaCl. The biological activity was detected in fractions eluted with 0.6-0.8 M salt concen-trations. EDF-H and EDF-L had a similar elution profile (data not shown). Following the above described purifications steps the inhibitory activity of EDF-H and EDF-L in the collagen gel contraction assay was the same. Serial dilutions indicated the presence of inhibitory activity in both at 1:80, but not at higher dilutions (data not shown). To evaluate whether the *in vitro* inhibitory effects of partially purified EDF(s) will translate into inhibition of wound contraction, they were applied to surgically inflicted (0.040 inch deep) wounds in pigs.

As documented in Fig 2., EDF(s) had striking effects on preventing wound contraction, when compared to control wounds treated with PBS. The difference was noticeable already 7 days after wounding but became more obvious at day 19. At this time the area resurfaced by new epithelium in wounds treated with EDF(s) was ~ 80% of the total surface area at time of wounding, compared to 64%

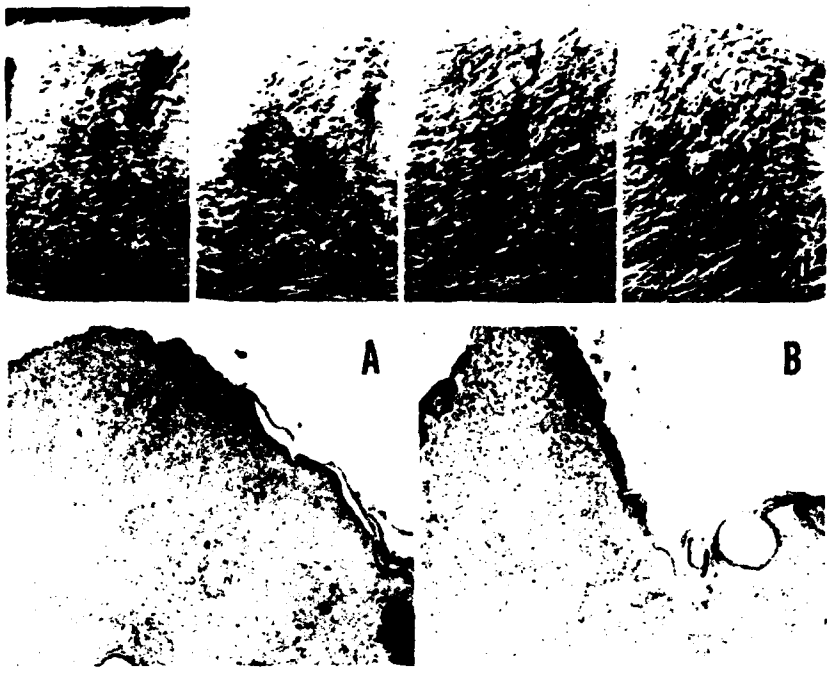

Fig. 2. The effects of EDF on wound healing (a-d). Clinical appearance of wound sites at 19 days, treated at time 0 with **(a)** EDF-L (1:8) ; **(b)** PBS; **(c)** Epidermal cell extract (1:400); **(d)** EDF-H (1:8); Note: The visible contraction of **(b)** in comparison of EDF treated wounds. **(A)** Histological appearance of EDF-L treated wound after 7 days, compared to **(B)** PBS control at the same time.

in the control wounds treated with PBS. Consequently the PBS treated wound contracted twice as much as wounds treated with EDF. Contraction inhibiting activity of the epidermal cell extracts and partially purified EDF(s) appeared to be the same. The clinical data therefore suggested that *in vitro* collagen contraction assay and *in vivo* wound contraction can be correlated. Evaluation of histological specimens taken 7 days after treatment of experimental wounds revealed that the density of fibroblasts in wounds treated with EDF(s) was ~ 1/10 of that found in wounds treated with PBS. In

contrast to the fibroblast inhibiting activity EDF-L also had an epidermal cell growth promoting activity. As shown in Fig 2A, in wounds treated with EDF-L the rete pegs were deeper and epidermis covering the wound was much thicker than in the control (PBS) treated wounds (See Fig 2B). The activity of EDF-L and EDF-H on growth stimulation of a subpopulation of epidermal cells was also detected in studies *in vitro*.

DISCUSSION

The experiments described here reveal that the partially purified EDF-H and EDF-L have an inhibitory effect on the contractility of fibroblasts *in vitro* and wound contraction *in vivo*. EDF(s) in addition had a stimulatory effect on the growth of epidermal cells both *in vitro* and *in vivo*.

Previous clinical observations of wound healing suggested, that epidermis may regulate dermal activity. It was noted, that following injury, migration into wounded area and proliferation of fibroblasts ceased when wounds became resurfaced by functional epithelium (Hunt et al, 1984). Moreover, substantial inhibition of wound contraction was observed in full thickness wounds transplanted with multilayered epidermal sheets grown *in vitro* (Eisinger, 1985). Thus, our findings of epidermal cell derived factors, shown to down regulate dermal activities, may furnish an explanation for previous clinical observations.

Throughout the process of purification of EDF(s), an *in vitro* assay of inhibition of contraction of three dimensional collagen gels was used. The assay was based on previous discovery that fibroblasts, in the presence of serum, induce contraction of collagen gels (Bell et al, 1983). From known growth factors, TGF-ß was shown to substitute for serum and markedly enhance the contraction normally observed in the presence of serum (Montesano, Orci, 1988). Under the same conditions platelet derived growth factor, basic

fibroblast growth factor and epidermal growth factor did not significantly change contraction of collagen gels or collagen sponges (Eisinger et al, 1988a), suggesting that TGF-ß is the most potent inducer and EDF an inhibitor of collagen contraction. The ability to contract a collagen matrix *in vitro* is believed to represent a fibroblast function that also operates *in vivo* during the process of wound repair (Ehrlich and Wyler, 1983). The cells responsible for contraction are the fibroblasts, particularly the so-called myofibroblasts characterized by myofibrils (Gabbiani et al, 1972). Wound contraction is of clinical importance, since it can reduce the amount of new tissue needed to reestablish organ integrity after tissue loss. However, this mechanism of wound closure is most desirable in mammals whose skin is loosely attached to underlying tissue. Human skin, with the exception of scalp, is closely knit to the tissues beneath it. Thus, wound contraction in human beings, if it occurred inappropriately, may lead to excessive scarring, constriction, immobilization and other disabilities.

We have shown good correlation between the *in vitro* inhibition of collagen gel contraction and inhibition of wound contraction *in vivo*, by EDF(s). Using both, *in vitro* and *in vivo* systems, studies toward understanding the mechanism of EDF(s) action can be initiated. Of particular interest will be to investigate whether EDF(s) can counteract TGF-ß's actions since both, *in vitro* and *in vivo* they elicit opposite biological effects. A prerequisite for detailed studies of EDF(s) is, however, the availability of sufficient amounts of purified factors.

The results presented here suggest, that more than one molecular form, found in epidermal cell extracts, exhibits the EDF biological activity. Even though, EDF-L and EDF-H share most biochemical and biological properties, they differ in their molecular size. Therefore, possibilities, that the 1 Kd EDF is a degradation product of the 30 Kd EDF, or that the 30 Kd peak

of activity results from aggregation of 1 or 5 Kd EDF(s), were considered. Use of inhibitors of proteolytic enzymes in the starting materials did not exclude the low molecular weight EDF(s). Similarly, treatment of 30 Kd EDF with different agents known to disaggregate proteins did not provide evidence for the second alternative. In view of current knowledge about other well studied growth factors, the finding of different molecular forms of EDF is not surprising. Particularly, members of TGF-ß and b-FGF families were shown to vary in their size, but not necessarily in function (Wozney et al, 1988; Gospodarowicz et al, 1987).

Because of clinical desirability for negative dermal and positive epidermal regulatory factors, and their value as tools for studies of growth regulation, our efforts toward detailed characterization of EDF molecules will continue. Elucidation of the factor's characteristics and mode of action should be of value both in understanding the normal mechanism of wound healing and, ultimately, in controlling its abnormal or clinically undesirable manifestations.

ACKNOWLEDGEMENT

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Enclosure 2b

Growth Factors and Other Aspects of Wound Healing:
Biological and Clinical Implications, pages 291-302

WOUND HEALING BY EPIDERMAL—DERIVED FACTORS: EXPERIMENTAL AND PRELIMINARY CLINICAL STUDIES

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INTRODUCTION

Epidermal cells form the outermost layer of skin and provide an important physiological barrier. When wounded, depending on the depth and the size of the area, regrowth of epidermis will occur from remaining hair follicles, glandular structures and from the edges of the wound. The clinical need to provide prompt functional coverage and prevent scar formation in deep and extensive wounds has led to the experimental use of tissue culture grown epidermal cells for wound healing (Eisinger *et al.*, 1980; O'Connor *et al.*, 1981; Hefton *et al.*, 1983; Hefton *et al.*, 1986). It was in the course of such studies that we (Eisinger, 1985) and others (Hefton *et al.*, 1983) noted that application of epidermal sheets grown in tissue culture accelerated the return to normal appearance of dermis and reduced or prevented scar formation.

In contrast to the well accepted regulatory effects of the dermis on epidermal cells, analogous effects of epidermal on dermis have not been studied extensively. Most research concerning growth regulation by epidermal cells has been concentrated on the epidermal inhibitors termed chalones (Bullough, 1962). It was believed that they might be the sole regulators of epidermal regeneration (Bullough, 1972). Our observations of epidermal wound healing have led us to the conclusion that epidermal cells might be the source of other important regulators of epidermal

behavior and also of dermal fibroblasts. To examine this possibility we used cell extracts and supernatant fluids from human and pig epidermal cells grown in tissue culture and examined their effects on wound healing in animals. Since epidermal cell extracts contained the same activity as supernatant culture medium, both were used in this study. We termed them collectively as Epidermis derived factors (EDF).

PROCEDURES

Preparation of Epidermal Cell Extracts and Culture Supernatants as Sources of EDF.

Human and pig epidermal cells were grown *in vitro* as described previously (Eisinger, 1985; Eisinger *et al.*, 1984). Confluent cultures (grown *in vitro* for 3-6 weeks) were washed twice with phosphate buffered saline (PBS), removed by scraping with a rubber policeman and pelleted at 180 g for 10 minutes. The pellet was resuspended in an equal volume of PBS, sonicated twice for 15 seconds and diluted 1:10 in PBS. The suspension was clarified by ultracentrifugation at 16,000 g and 150,000 g. The resulting extract, to be considered as undiluted starting material, was aliquoted and frozen at -70°C. For collection of secreted factor(s), epidermal cells grown to confluency were washed with PBS and fed with medium containing no fetal calf serum. The supernatants were collected after 24 and 48 hours.

Wound Healing Assay

Domestic outbred swine were anesthetized with ketamine hydrochloride, intubated and maintained on a mixture of halothane, nitrous oxide and oxygen. The operative site was shaved and washed twice with Betadine and 70% alcohol. Wounds 0.040 inch deep were created on the thorax with a Brown dermatome. Non-adhesive dressing (Release, Johnson and Johnson, New Brunswick, NJ) was cut to fit the wound, soaked in the materials to be tested and applied to the wound bed. Control wounds were covered with Release soaked in PBS. The dressing was covered by multiple layers of gauze held in place by silk ligatures and protected with Elastoplast

bandage. After surgery the pigs received analgesics to control pain. The wounds were examined at 2-4 day intervals and 3mm punch biopsies were taken from the center of the lesions. The wounds were redressed after 5 days with Release soaked in Ringers solution. After 12-14 days the wounds were left uncovered.

Histology

The punch biopsies were fixed in Bouin's solution overnight, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and evaluated by light microscopy. The distance between the nuclei of adjacent fibroblasts was measured in the center of the new granulation tissue using a calibrated occular and the fibroblast density was calculated. The epidermal cell population of the same slides was evaluated by counting the number of cell layers arising from hair follicles within the new granulation tissue.

Clinical Treatment of Horses and Dogs

Five horses suffering from long-term (up to 6 months) non-healing wounds on the distal part of the limbs, four of which exhibited exuberant granulation, were used for clinical studies. In three cases the excess granulation tissue was removed surgically under general anesthesia, in one case under regional analgesia and in the fifth case the wound surface was debrided of necrotic material. Following surgery the wounds were covered by Release soaked in pig EDF diluted 1:20, and held in place by gauze under Elastoplast. They were treated over 10 days by three sets of dressings soaked in EDF. Three dogs suffering from destructive ulceration of the perianal and pudendal regions were sedated with acetylpromazine and the ulcerated area was debrided of necrotic material. A ring-shaped pad of Release soaked in 1:20 EDF was cut to fit the ulcerated area, covered by a ring of polyethylene film and held in place by adhesive strips. The dressing was replaced twice at three day intervals.

Test materials compared with EDF were as follows: Epidermal growth factor (EGF) was purchased from Collaborative Research Inc., Bedford, MA, and transforming

growth factor α (TGF α) from Peninsula Laboratories, Inc., Belmont, CA. Urogastrone was a generous gift from Dr. K. McCullagh.

Physiological Healing of Surgically Inflicted Wounds in Pigs

The surgical removal of 0.040 inch thick skin, using a Brown dermatone, created wounds void of all epidermis and superficial dermis. However, deep dermis containing hair follicles and sebaceous glands remained. Wounds dressed immediately with a non-adhesive dressing soaked in phosphate buffered saline were covered by a scab within 24 hours. No appreciable epidermal growth was seen for the next 6-7 days. On the eighth day epidermis was moving in from the edges of the wound and from epidermal remnants. At this time, the surface of the wound showed continuing signs of inflammation and some degree of exudation. Contiguous epidermal coverage was usually observed 10 to 12 days after injury. Biopsies taken from the center of the wound 5 days after injury have shown extensive connective tissue growth, high degree of vascularity and many inflammatory cells on the unhealed surface. There was virtually no evidence of epithelial activity. By day 8, a loosely attached layer of epidermal cells, uneven in thickness, was observed. The granulation tissue had a highly active appearance and contained many large dilated blood and lymphatic vessels. By day 12 there was a multilayered sheet of epidermis which was still loosely covering a thick layer of granulation tissue. Twenty three days after injury the epidermis of such wounds was firmly attached. It contained many mitotically active cells within the basal layer. The epidermal layer was thicker than in normal skin and the granulation tissue was very deep and highly cellular.

Enhanced Re-Epithelialization by Epidermal Derived Factor (EDF).

In wounds treated with EDF the clinical picture was strikingly different from that of the control wounds. Epithelial growth was clearly visible by day 3 and the inflammatory reaction was less pronounced than in the control wounds. By day 5 epithelialization was well advanced and hair growth in the area was noticeable. By day 10 the gross difference between EDF treated and control wounds were less clear and by day 15 had

disappeared, except that the hair in the treated areas was longer and more abundant. Biopsy specimens taken from EDF-treated wounds five days after wounding have shown numerous branches of proliferating epidermal cells with many mitotic figures. The epidermal cells have penetrated the new granulation tissue and spread in a thin layer over its surface, under the crust of exudate. By day 8 there was a contiguous multicellular hyperplastic layers of new epidermal cells. By day 12 the rete pegs were shorter and their connections to the deep epidermal structures were lost. By day 23 the EDF treated wounds were covered by epidermis resembling non-wounded skin.

Suppression of Hyperproliferation of Fibroblasts by EDF

Evaluation of biopsies taken from the wounded areas 5, 8, 12 and 23 days post-wounding revealed that the physiological response to wounding was marked by an increase in the number of fibroblasts and other inflammatory cells in the newly formed granulation tissue. In the wounds treated with EDF, the hyperproliferative response was markedly decreased, but new collagen matrix strands became apparent 8 days post-wounding. By day 12, the depth of the new granulation tissue in the EDF treated wounds was substantially less than in the controls.

The Gradient Effect of Growth Factors

To establish the optimal conditions for the evaluation of effects of different growth factors and the optimal dose of the factor(s) to be used in such studies, the lateral diffusion of EDF in an experimental wound was evaluated. As shown in Figure 1, a wound 11.5 cm long and 5 cm wide was created. Seven centimeters of this area was covered by Release soaked in EDF (diluted 1:10) and the rest of the wound was covered by Release soaked in PBS. An additional wound 4.5 cm long was covered by Release soaked in PBS (control). Biopsy specimens taken at 5, 8, 12 and 23 days after wounding have shown, that epidermal growth was enhanced and fibroblast density suppressed in the treated wound (designed EDF, Gradient I and II) in comparison to the control wound. Stimulation of epidermal cell growth was most profound in the area designed Gradient I (Figure 2).

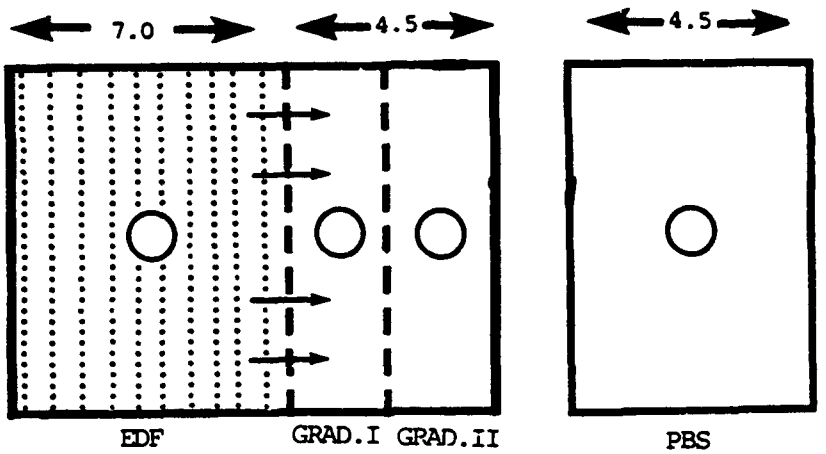

Figure 1. A schematic presentation of the wounds for studies of the lateral diffusion of growth factors (Gradient effect). Surgically prepared wounds (0.040 inches deep 11.5 x 5 cm and 4.5 x 5 cm were covered by non-adhesive dressing. The dotted area was covered by Release soaked in EDF diluted 1:10 (EDF); the rest of the wound was covered by the same non-adhesive dressing soaked in phosphate buffered saline (GRAD I and GRAD II); as was the control wound (PBS). Biopsies were taken from the center of the different areas (0).

This area also had the least density of fibroblasts per cm2 (Table 1).The experiment therefore suggested that optimal effect of EDF is at a higher dilution of the factor than 1:10. In a series of experiments (data not shown) we have found, that optimal effect of EDF was at a dilution 1:20 - 1:40, when the material was derived from epidermal cell extracts. In addition we tested lateral diffusion in separate wounds, located at 1 cm distance from each other. It was found that a high concentration of the factor in one wound can influence the healing tissue in the next ones. Therefore proper spacing of wound beds for evaluation of the effects of different growth factors is of paramount importance.

Comparison of the Effects of EDF and Other Known Growth Factors on Surgical Wounds

Other known growth factors, previously shown to enhance epithelialization and to stimulate connective tissue formation,

Figure 2. Light micrograph of histological sections from biopsied areas shown in Figure 1.
(a) (EDF). Arrows point to the hyperplastic epidermis.
(b) (GRAD I). Arrows indicate epidermal sprouts, originating from hair follicles. Note the low cellularity in the granulation tissue. c) (GRAD II). The epidermal sprouts are shorter and there is an obvious increase in the number of fibroblasts and lymphocytes in the dermis. d) (PBS). The control wound is not yet covered by epidermis and the granulation tissue is highly cellular. (Eight days post-treatment, magnification x 200).

were tested in the above experimental system. Epidermal growth factor (EGF), urogastrone and transforming growth factor (TGF-α), all used at a concentration of 200 ng/ml, stimulated re-epithelialization in comparison to the control wounds. However, proliferation of cells from remaining hair follicles and glandular structures was less striking compared to wounds treated with EDF. Biopsies taken 8 days after initial treatment revealed that in EGF treated wounds the epidermal sprouts had 30 layers and the top was covered by a single layer of epidermal cells. A similar picture was seen in wounds treated by urogastrone. TGF-α treated wounds were covered by four layers of epidermal cells, but no obvious sprouts were present. At the same time EDF treated wounds were covered by seven layers of epidermal cells and the sprouts were up to 80 layers thick. The density of fibroblasts in wounds treated with EGF, urogastrone and TGF-α were 0.6, 1.2 and 1.6 x 10^6 cells/cm^2, respectively. The density of fibroblasts in EDF treated wounds was 0.11 x 10^6, similar to that of the unwounded skin (0.06 x 10^6) while the control wounds had 1.4 x 10^6 cells per cm^2. EDF treated wounds had 6-16 times less cells in the new granulation tissue than control wounds or

wounds treated with EGF, urogastrone or TGF-α. The most effective stimulation of fibroblasts was by TGF-α.

	EDF (1:10)	GRAD. I	GRAD. II	PBS
FIBROBLAST DENSITY CELLS/cm^2	0.6×10^6	0.33×10^6	0.95×10^6	1.4×10^6
EPIDERMAL CELL LAYERS (top/sprouts)	4/18	4/28	2/6	0-1/0

Table 1. Numerical evaluation of the fibroblasts and epidermal cell layers as shown in Figure 1 and Figure 2. The evaluation is based on serial histological sections and was calculated as described in the text.

CLINICAL STUDIES

Horses with severe lacerations of the metacarpal region or with a non-healing traumatic ulcer were treated with EDF (1:20). The wounds were long standing (more than 6 months) and their general feature was one of recurrent granuloma. Out of five horses treated, four responded by growth of new epidermal cells from the edges of the wound and from isolated epidermal remnants. In one case new growth was visible by day 4 and in the others by day 6 of the treatment. Complete epithelialization of the lesions took between 17 and 35 days which reflected the variation in size of the area to be covered by epithelium. A common feature of all five cases was that whether or not the epithelium was stimulated to grow, the excessive proliferation of granulation tissue was clearly suppressed by the EDF treatment. None of the four lesions that responded to treatment has recurred in more than a year.

Three long established (more than six months) cases of perianal ulceration of dogs were treated by application of EDF-

soaked dressings over periods of 10 days. Two of these responded favorably and the ulcerated areas epithelialized over the following 24 and 35 days respectively. In the third case there was an initial appearance of epidermal stimulation but this was transitory and destructive ulceration continued after an intermission of about 7 days.

DISCUSSION

The findings described here suggest that there may be an important role for locally produced factor(s) in both the positive and negative feedback mechanisms and in the maintenance of homeostasis in the epidermis and dermis. The autocrine regulators previously identified in epidermal cells (chalones) have been found to be potent inhibitors (Bullough *et al.*, 1967; Bullough and Laurence, 1964). This has led to the conclusion that epidermal cell growth is regulated by negative feedback. Furthermore, factors that have been shown to stimulate epidermal cell growth, such as epidermal growth factor, its human analog urogastrone and closely related TGF-α, have all been isolated from tissues other than epidermis (Savage and Cohen, 1972; Starkey *et al.*, 1975; Anzano *et al.*, 1983). However, the findings presented here indicate that epidermal cells themselves are capable of producing a factor or factors that stimulate local proliferation of epidermal cells and inhibit the migration or proliferation of fibroblasts *in vivo.* The inhibitory effect of these factors on fibroblasts cannot be attributed to chalones, as by definition they inhibit only the tissue of origin (Iverson, 1981; Holley *et al.*, 1980).

The possible roles of different growth factors in wound healing are currently of general interest. It has long been conjectured that EGF must play a part in wound healing since it is present in saliva, and animals naturally lick their wounds (Naill, 1982). Application of EGF and TGF-α to experimental wounds has been shown both to accelerate re-epithelialization and to promote connective tissue formation (Brown *et al.*, 1986; Laato *et al.*, 1986; Shultz, 1987). Transforming growth factor-β accelerated accumulation of protein, collagen and DNA in wound chambers in rats (Sporn *et al.*, 1983). In contrast to all the above growth

factors shown to stimulate fibroblast proliferation, EDF had a mild suppressive effect on fibroblasts *in vivo*. It also inhibited by 95% fibroblast induced collagen contraction *in vitro* (Eisinger *et al.*, 1987). Epidermal growth factor, fibroblast growth factor, platelet derived growth factor, transforming growth factor-β, nerve growth factor and extracts of WI - 38 cells did not have this inhibitory activity (Eisinger *et al.*, 1987). The fibroblast inhibitory activity of EDF has proven useful in clinical treatment of granulomatous wounds in horses, but not in deep ulcerated wounds, lacking connective tissue, in man (Carter *et al.*, 1987). Therefore, use of different growth factors, individually or in combinations, will have to be carefully chosen depending on the type of the wounds to be treated. It seems plausible that the experimental gradient system described here would lend itself for studies on dose dependent combined effects of different growth factors.

Since combined effects of different factors may result in synergism, agonism or antagonism, the next step in the analysis of the epidermal derived factors is to define whether the dual biological activity is associated with the same or different molecules. Experiments so far have shown that both properties can be abolished by acid treatment, but can resist heat (60°C) and alkaline treatment. Another factor (TGF-β) has been shown to have either growth promoting or growth suppressing effects (Roberts *et al.*, 1985; Fine and Goldstein, 1987; Tucker *et al.*, 1984) on different cell types. It may therefore be that epidermis produces a molecule responsible for both positive and negative regulatory effect in the skin.

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Enclosure 3

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INFLUENCE OF AN EPIDERMAL CELL EXTRACT ON SKIN HEALING AND SCAR FORMATION

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Summary: *We have examined the possible regulatory role of epidermal cell extract(s) (ECE) on skin cells, namely fibroblasts and keratinocytes, both* in vivo *and* in vitro *with particular reference to modification of scar formation. In an experimental wound model in pigs, it was found that extracts of cultured human and pig keratinocytes stimulated replication of epidermal cells and their migration from wound edges and remnants of hair follicles and sebaceous glands, together with hair growth, but at the same time suppressed fibroblast proliferation in the dermis. Sections of healing skin wounds that had been treated with ECE showed the presence of a thick layer of epidermal cells lying on relatively sparse dermis. There was little or no contraction in treated wounds and scarring was minimal. Clinical studies of granulomatous lesions of horses and ulcerated wounds in dogs that had been treated with ECE supported these findings. In contrast to its apparently general stimulation of keratinocytes* in vivo, *ECE had a highly selective effect* in vitro *on epidermal cells plated at low density in the absence of a feeder layer, which suggests that its action* in vivo *may be confined to a specific sub-population of rapidly proliferating keratinocytes or alternatively mediated through a second messenger from another type of cell. The inhibitory effect of epidermal cell extract on fibroblasts* in vitro *was shown by its ability to prevent the contraction of collagen sponges by fibroblasts. These results suggest an important role for epidermal factors in the growth regulation of both epidermal and dermal cells during wound healing.*

Introduction

There are many factors involved in the healing of wounds and the subsequent degree of scarring, which include nutrition, age, site and type of injury, contamination with micro-organisms, presence of foreign bodies, ambient temperature and surgical or medical treatment. The clinical requirement for rapid coverage of large open wounds has led to the development of various types of skin grafting, and more recently to the use of epidermal cell sheets grown in tissue culture. It is well established that these techniques can prevent the formation of excessive granulation tissue and reduce the probability of substantial wound contraction and extensive scar formation. During the last few years there has been much interest in the possible clinical applications of various natural or artificial growth factors, a rapidly increasing number of which are being identified. Most appear to be highly specific in their target cells, and interest

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has centred chiefly on their role in stimulating epidermal activity. The possible interactions of epidermis or epidermal-derived growth factors with *dermis* have been largely ignored. This paper describes some preliminary observations on the use of an epidermal-derived extract that can be harvested from cultures of human or pig epidermal cells and which enhances epidermal growth *in vivo* and *in vitro*. It also has the property of suppressing the growth of fibroblasts and of inhibiting their contractile behaviour *in vitro*. When applied to extensive clinical or experimental skin wounds *in vivo* it reduces granulation-tissue formation and subsequent scarring in the dermis at the site of injury.

Methods

Cell extracts and supernatant from human or pig keratinocytes grown in primary culture were collected and treated under sterile conditions as described by Eisinger *et al.* (1, 2). Standard, superficial experimental wounds, 1.0 mm deep, were created with a Brown dermatome on the backs of commercial, outbred pigs anaesthetized with ketamine, halothane and nitrous oxide/oxygen. The lesions were covered with non-adhesive dressings (Release®, Johnson and Johnson, NJ, USA) soaked in the materials to be tested and held in place under gauze padding by ligatures and adhesive tape. In control wounds the dressings were soaked in phosphate-buffered physiological saline (PBS). Punch biopsies of the wound bed were made at 2- to 4-day intervals and the wounds redressed at 5 and 10 days but left open and uncovered after 12–14 days. The biopsies were fixed and processed by standard histological techniques and sections cut at 5 µm for microscopical examination.

Clinical investigations were carried out on chronic, non-healing granulating wounds in five horses and on perianal and other ulcers in domestic dogs. The lesions were cleaned and treated with Release dressings soaked in ECE at a concentration of 1:200 over a period of 10 days. The dressings were replaced at 3-day intervals. In three cases in the horses, excess granulation tissue was removed surgically before the ECE treatment was started. This had been done previously but the wounds had not healed and granulation tissue had regrown out through the wound surface, a feature which is common in wounds on the distal parts of the legs of horses. Healing was assessed by observation and measurement of the position of the epidermal ingrowth over the wound surface and calculation of the area of granulation tissue that remained exposed.

Comparative observations were made on an experimental wound model in pigs using Release dressings soaked in solutions of other putative growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF) and platelet-derived growth factor (PDGF).

Laboratory tests of the effects of the ECE *in vitro* were carried out on dissociated cultures of pig and human epidermal cells and on collagen sponges seeded with human or pig skin fibroblasts (3). Epidermal cell growth and collagen sponge size was measured daily by microscopical observation using a calibrated eyepiece.

Results

Experimental wounds

In the control, untreated wounds there was the usual progression from haemorrhage to scab formation over 24 h, and little epidermal proliferation was visible for about 6 days although it had become very obvious by day 8. Complete wound coverage was usual by day 12. This pattern was confirmed by the histological findings which also

showed that some degree of inflammation persisted in the healing surface of the untreated, control lesions until after epithelialization was complete.

In the ECE-treated wounds the gross and microscopic picture was markedly different. Epithelial regrowth was clearly visible by 3 days after wounding and the inflammatory response less pronounced. Epithelial coverage was well advanced by 5 days and there was a very striking growth of hair from residual hair follicles in the wound bed. The wound surface was usually completely covered by epithelium from 6 days onwards. By 10–15 days there was little difference in appearance between treated and control wounds except that the treated wounds *a*) had a much thicker epidermal covering, *b*) had less residual inflammatory reaction and c) had far greater hair growth. Histological examination of biopsies from the wounds confirmed that epithelialization began earlier and was more vigorous in the ECE-treated wounds than in the controls, and the inflammatory cells disappeared earlier.

A striking feature was the relatively small amount of new granulation tissue which grew on the wound surface, although initially it was well vascularized before it became covered by epidermis. This new connective tissue was somewhat sparsely populated by fibroblasts and its vascularity was short-lived, with an early reduction in numbers of patent, perfused small vessels, when the epidermis covered it. By contrast, the untreated control wounds showed a much greater amount of connective-tissue growth, more prolonged neovascularization and slower coverage with new epidermis. In the treated wounds the epidermis was considerably thicker than that of controls in the early stages of healing, and there was marked cellular activity in the hair follicles associated with growth of hair and epidermal proliferation. By the 23rd day there was little difference between the treated and control wounds, except that the latter still showed some epidermal mitotic activity, and the greater amount and cellularity of new connective tissue generated during the early stages of healing were very evident.

Random counting of the fibroblast population in the two groups showed that the density of cells was at least an order of magnitude lower in the ECE-treated wounds ($0.11 \cdot 10^6 \cdot cm^{-2}$) as compared to the numbers in the control, PBS-treated lesions ($1.4 \cdot 10^6 \cdot cm^{-2}$). This contrasts with results obtained using some other growth factors such as EGF, urogastrone and TGF where the fibroblast populations in healing and recently-repaired dermis were large. These results from the fibroblast population in the experimental pig wounds correlated well with observations *in vitro*. ECE inhibited fibroblast-induced shrinkage of collagen sponges and limited fibroblast proliferation in culture, while the other growth factors tested did not. It was more difficult to demonstrate effects of ECE on epidermal cells in culture and it became clear that the enhanced growth of epidermis provoked *in vivo* was either the expression of stimulation of a particular subclass of keratinocytes or was being relayed through a second effector pathway. In cultures of isolated epithelial cells, ECE caused proliferation of certain individual cells only, to form aggregate colonies of 50 or more cells. The great majority of cells in the colony did not appear to be affected directly by the presence of ECE.

Accidental clinical injuries in horses

Four horses with severe lacerations of the metacarpal region, that had resulted in exuberant granulation tissue protruding from the wound site (a frequent clinical problem in the distal parts of the limbs of horses), were treated over ten days with 3 sets of dressings soaked in a solution of 1:200 ECE, starting immediately after removal of the excess tissue in three of the cases. Previous excisions had been performed, but healing had

not occurred. In a fifth case suffering from an indolent non-healing ulcer of traumatic origin, necrotic tissue was debrided from the lesion before ECE treatment was applied. In four of the horses, including that with the ulcer, new epithelial growth appeared from the edges of the wounds, in one case at day 4 and in the others at day 6. In the fifth horse there appeared to be no grossly detectable epidermal response to the treatment. Complete epithelialization of the wound surfaces that responded took between 17 and 35 days, which reflected the variation in size of the areas to be covered. A common feature of all five cases was that connective-tissue regrowth was suppressed, even in the one where there was no satisfactory epidermal response, and subsequent scar formation, despite the chronic nature of the injuries, was minimal.

Spontanous ulcerations in dogs

One of the more intractable complications of impaction of the perianal sacs in dogs is chronic, progressive destructive ulceration of the circumanal region. Three long-established (< 6 months) cases of perianal ulceration were treated by application of ECE-soaked Release dressing over a period of 10 days. Two of the three responded favourably and healed in 24 and 36 days respectively. In the third case there was an initial appearance of epidermis at the wound edge, but this was transitory and destructive ulceration recurred after about 7 days. None of the clinical lesions described above as having responded to treatment have recurred during the past 18 months, and the post-repair scarring (and anal stricture in the dogs) which would have been expected has been minimal.

Discussion

The experimental and clinical cases described here show that epidermal cells can produce a factor(s) that not only stimulates epidermal growth but also reduces fibroblast proliferation and possibly migration, diminishes scar formation, and limits would contracture through inhibition of fibroblast contraction. The findings indicate a role for locally-produced factors in encouraging and suppressing various cellular activities associated with skin wound healing, and may provide some explanation for common observation that the completion of epidermal covering of wounds normally suppresses further connective-tissue growth. Previous work has established the presence of a negative feedback system in the control of epidermal cell growth through chalone production by mature cells (4). It has also been suggested that EGF plays a role in normal wound healing due to its presence in saliva and because the licking of wounds is the natural response of mammals to injury, while it is also known that EGF and TGF can promote general growth in epidermal cells *in vitro*. However, these factors also tend to increase connective-tissue formation when applied to healing wounds (5).

The factor described here is on the other hand a suppressor of fibroblast activity and, at least *in vitro*, a selective stimulant of epithelial cells. *In vitro* this selectivity is not so apparent and it may be speculated that the factor could be acting through a second messenger in stimulating some types of epithelial cells, although the effect on fibroblasts seems direct both *in vivo* and *in vitro*.

A notable feature of connective-tissue growth in wounds treated with ECE was the rapid appearance of relatively large collagen strands, as compared to the fine fibrils usually seen in granulation tissue. A rapid increase in Type III collagen is characteristic of most healing wounds, while Type III and Type V are commonly present in hypertrophic scars (6). It is possible that the reduced or absent wound contracture and scarring that followed ECE application might be due to early, direct synthesis of Type I collagen by resident,

non-proliferating fibroblasts. In the normal sequence of connective-tissue healing, Type III collagen synthesis, which is predominant initially, is followed by remodelling and scarring. A feature of the wounds treated with ECE was the minimal formation of scar tissue and the promotion of rapid re-epithelialization even after a period of indolence. This suggests that the material may have a useful role to play in the treatment of non-healing wounds or other indolent lesions, or of those conditions in which disfiguring or excessive scar formation may be a sequel. The marked stimulation of hair growth by ECE also offers interesting cosmetic possibilities, as does the finding that the factor(s), like chalones, appears to be cross-specific in its activity.

It is not yet clear whether ECE is a single factor or a closely linked combination of related molecules, but both epidermal stimulating and fibroblast suppressing activities are lost after acidification yet survive heating to 60°C and alkaline treatment. Since another factor, TGF, is known to have both growth stimulating and inhibiting properties depending on the test cell type, it is equally possible that ECE is a single substance with more than one biological action.

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Enclosure 4

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Medical Sciences

Growth regulation of skin cells by epidermal cell-derived factors: Implications for wound healing

(re-epithelization/keratinocytes/fibroblast inhibition)

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ABSTRACT **Epidermal cell-derived factors (EDF), present in extracts and supernatant fluids of cultured epidermal cells, were found to stimulate the proliferation of keratinocytes but to inhibit fibroblasts. *In vitro*, the effect of EDF on epidermal cells resulted in an increased number of rapidly proliferating colonies composed mainly of basal keratinocytes. Control cultures grown in the absence of EDF had a high proportion of terminally differentiated cells. In fibroblast cultures EDF inhibited the ability of fibroblasts to cause contraction of collagen sponges by 90%. Epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor β, nerve growth factor, and extracts of WI-38 cells (human embryonic lung fibroblasts) did not have this inhibitory activity. Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes from remnants of glands, hair follicles, and wound edges. The restoration of complete epidermal coverage of wounds treated with EDF occurred twice as rapidly as that of control wounds. In addition, regenerating dermis in the EDF-treated wounds contained 1/5th to 1/15th as many cells as wounds treated with epidermal growth factor, urogastrone, transforming growth factor, or phosphate-buffered saline. The use of EDF to enhance re-epithelization and to prevent scar formation is proposed.**

Epidermis, the outermost layer of the skin, consists of stratified squamous epithelium, which differentiates from proliferating basal keratinocytes. Beneath this is the dermis, whose main representative cell type is the fibroblast. The functional role of the dermis in growth regulation of the epidermis *in vivo* has been documented previously (reviewed in ref. 1). Supportive evidence for the importance of dermal cells in aiding the growth of epidermal keratinocytes has come from *in vitro* studies initiated by Green *et al.* (2). These studies have shown that epidermal cells can be grown in tissue culture on a feeder layer of 3T3 fibroblasts even at cloning densities (3). Under such conditions epidermal cell growth can be potentiated by epidermal growth factor (EGF) (3) or cholera toxin (4) in the growth medium. In contrast, without a feeder layer, epidermal cells grew successfully *in vitro* only when seeded at higher densities (5) despite the presence of EGF. Attempts to grow epidermal cells at cloning densities in the absence of feeder layers resulted in colonies consisting mainly of differentiated keratinocytes and adventitious colonies of fibroblasts. The rapid proliferation of fibroblasts resulted in the eventual overgrowth of such cultures. It thus appeared that the successful growth of keratinocytes seeded at high densities might be due to the presence of a growth factor or factors produced by epidermal cells themselves. We therefore prepared epidermal cell extracts and supernatant fluids from epidermal cells grown *in vitro* and studied their effects on keratinocytes and fibroblasts *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Preparation of Epidermal Cell-Derived Factor(s) (EDF). Human and pig epidermal cells were grown *in vitro* as previously described (6). Confluent cultures (grown *in vitro* for 3–6 weeks) were washed twice with Dulbecco's phosphate-buffered saline (PBS), removed by scraping with a rubber policeman, and pelleted at 180 × *g* for 10 min. The pellet was resuspended in an equal volume of PBS, sonicated twice for 15 sec, and diluted 1:10 in PBS. The suspension was clarified by two consecutive ultracentrifugation steps at 16,000 × *g* for 20 min and 150,000 × *g* for 45 min. The resulting clarified epidermal cell extract, considered concentrated starting material, was divided into aliquots and frozen at −70°C. To collect the secreted factor, epidermal cells grown to confluency were washed twice with PBS and re-fed with half the usual volume of medium, containing no fetal calf serum. The epidermal cell supernatants were collected 24 and 48 hr later. The protein concentrations of epidermal cell extract and epidermal cell supernatant were 0.26 mg/ml and 0.04 mg/ml, respectively. Because both epidermal cell extract and epidermal cell supernatant contained the same biological activity we termed them collectively epidermal cell-derived factor(s) (EDF).

Stimulation of Growth of Epidermal Cells *in Vitro*. An epidermal single-cell suspension was prepared as previously described (6). Cells were seeded at 7.5×10^4 cells in a 12-well plate (Costar, Cambridge, MA) or 5×10^5 cells in a 60-mm Petri dish in minimal essential medium with Earle's salts, containing nonessential amino acids, 2 mM L-glutamine, antibiotics, and 10% fetal bovine serum (cMEM). For keratinocyte cultures the medium was adjusted to pH 6.5 with 1 M hydrochloric acid. Test samples of putative growth factors at different dilutions were added at the time of plating or 24 hr later. The medium was changed at 3-day intervals. After 12 days of incubation at 36°C the cells were fixed with buffered formaldehyde and stained with hematoxylin and eosin. Numbers of colonies and numbers of cells per colony were counted under the microscope.

Measurement of Fibroblast Contractility *in Vitro*. Pig or human fibroblasts were derived from normal skin and grown in tissue culture in cMEM. Cells passaged four to eight times were used for the experiments. Collagen sponges 2 mm thick made of type I bovine collagen were purchased from Biomaterials Center (Piscataway, NJ). These were cut into disks 3.2 cm in diameter and placed into six-well plastic dishes

Abbreviations: EDF, epidermal cell-derived factors; EGF, epidermal growth factor; NGF, nerve growth factor; TGF-α and -β, transforming growth factor α and β; basic FGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor.

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(Costar) and 0.8×10^6 cells were seeded per 8-cm^2 area in cMEM. Six hours later the medium was changed and substances to be tested were added in cMEM. Shrinkage of collagen sponges was measured daily, as described previously (7). The experiments were terminated 3 days after addition of factors. Inhibition of collagen contraction was calculated from the ratio of percent contraction of triplicate samples containing factor and control samples in the absence of factors. To account for the variability in different batches of collagens, values obtained for spontaneous contraction of collagens soaked in tissue culture medium were subtracted from all samples tested. For the final evaluation values obtained for 100% contraction of control samples represented 0% inhibition of contraction.

Assay of EDF Activity *in Vivo*. Domestic outbred swine were anesthetized with ketamine hydrochloride, and anesthesia was maintained by a mixture of halothane, nitrous oxide, and oxygen. The operation site was shaved and cleansed with Betadine and 70% (vol/vol) ethyl alcohol. Wounds 0.040 inch (1 mm) deep were created on the sides of the thorax with a Brown dermatome. Nonadhesive dressing (Release, Johnson & Johnson) was cut to fit the size of the wound, soaked in the materials to be tested, and applied to the wound bed. The control wounds were treated with Release soaked in PBS. The dressing was covered with multiple layers of gauze held in place by silk ligatures and protected by an Elastoplast bandage. After surgery the pigs received analgesics such as Tylenol to alleviate discomfort. The wounds were observed at 2- to 4-day intervals and 3-mm punch biopsy specimens were taken from the center of the wound. After 5 days the wounds were redressed with Release with Ringer's solution only. At 12–14 days after surgery wounds were usually left uncovered.

Histology. The 3-mm punch biopsy specimens were fixed in Bouin's solution overnight and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and evaluated by using a light microscope. The central area of the new granulation tissue in a section was selected, the distance between the nuclei of adjacent fibroblasts was measured by using a calibrated ocular, and the fibroblast density was calculated. The epidermal cell population of the same section was evaluated by counting the number of epidermal cell layers arising from hair follicles within the new granulation tissue and covering the surface.

Other Supplies. EGF and nerve growth factor (NGF) were purchased from Collaborative Research (Waltham, MA). Transforming growth factor α (TGF-α) was purchased from Peninsula Laboratories (San Carlos, CA). Transforming growth factor β (TGF-β), basic fibroblast growth factor (basic FGF), and urogastrone were generous gifts from R. K. Assoian (Columbia University, New York), D. Gospodarowicz (University of California, San Francisco), and K. McCullagh (British Biotechnology, Oxford), respectively.

RESULTS

EDF-Dependent Growth Stimulation of Epidermal Cells *in Vitro*. When seeded in cMEM at a density of 1.8×10^4 cells per cm^2 in the absence of a feeder layer, epidermal cells attached and in 12 days gave rise to small colonies, mostly composed of $\approx$100 cells. Larger colonies, composed of more than 100 cells, arose at a frequency of 4 per 5×10^5 cells plated. These colonies contained about 50% small undifferentiated cells and an equal proportion of large terminally differentiated cells (Fig. 1*A*). In the presence of epidermal cell extracts diluted 1:80, or supernatant fluids from epidermal cell cultures diluted 1:5, there was a significant difference in the numbers and the morphology of the cells composing such colonies. The number of cells in individual colonies was increased 3- to 30-fold, and they were composed entirely of basal keratinocytes. Occasionally, more differentiated cells were found on the edges of such groups (Fig. 1*B*). The number of colonies containing more than 3000 cells was relatively small, 15 or 16 colonies in a 35-mm Petri dish. However, cells in such colonies continuously divided, and cell numbers per colony reached up to 1.2×10^4 cells in 20 days. The effect of epidermal cell supernatant and epidermal cell extract was concentration dependent, and half-maximal activity of epidermal cell supernatant was at a dilution of 1:5, whereas that of epidermal cell extract was at 1:80 (data not shown). When cultures were seeded at a higher density (2×10^5 cells per cm^2) the effects of EDF

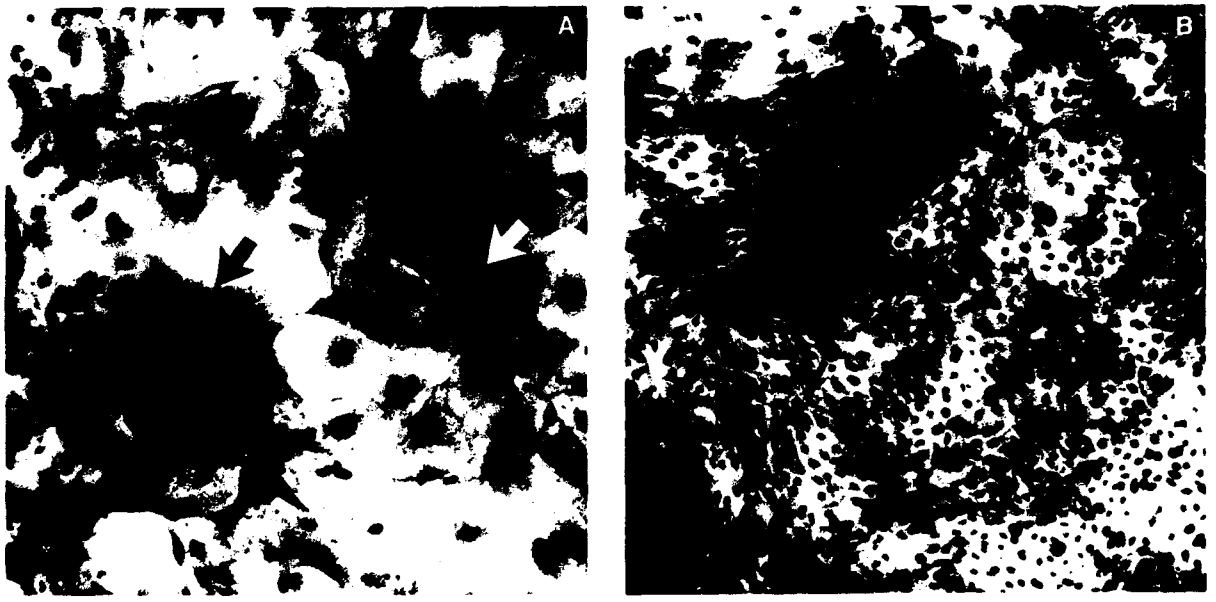

FIG. 1. Morphology of human keratinocytes within colonies grown from single-cell suspensions (at 12 days in culture). (*A*) Keratinocytes grown in culture medium in the absence of EDF. The colony is composed almost entirely of differentiated keratinocytes. Arrows indicate small clusters of basal keratinocytes. (×110.) (*B*) Keratinocytes grown in the presence of epidermal cell supernatants diluted 1:5. Note that the colony is composed of small cells with a relatively large nucleus. Arrows indicate the cells in mitosis. A few differentiated cells can be seen at the edge of the colony. (×110.)

were not detectable either by cell counts or by [^{3}H]thymidine incorporation, most probably because of saturation by factors produced by the epidermal cells present in the culture. In addition to the growth-stimulating effects of EDF on keratinocytes, obvious inhibition of fibroblast growth was seen in cultures seeded at lower densities in the presence of EDF. The same inhibitory effect was seen when the cultures were seeded at high densities. In the absence of EDF 1 or 2 colonies of fibroblasts were found 12 days after plating with epidermal cell suspension at lower densities (1.8×10^4 cells per cm^2). To compare the effect of EDF with the growth-promoting activity of EGF we used the tissue culture system described by Rheinwald and Green (2). While EGF stimulated growth of epidermal cell colonies as previously described (3), EDF had no effect, suggesting that EDF and [illegible] do not have the same biological activity.

[illegible]ts of EDF on Fibroblasts *in Vitro*. Fibroblasts seeded [illegible]agen caused, in the first 3–4 days, up to a 50% decrease in the original size of the collagens. After this initial contraction the size of collagens remained constant for the next 4 days. In the absence of fibroblasts, collagen sponges soaked in the medium decreased in size only minimally (between 2% and 10%) in the first 3 days. Therefore, the time for evaluation of collagen contraction was set at 72 hr after the addition of the tested materials. Fig. 2 *A* and *B* shows the effects of EDF and other growth factors on the inhibition of fibroblast-induced collagen contraction. EDF had the highest inhibitory activity of all substances tested. This activity was detected in pig or human epidermal cell extracts as well as supernatant fluids of cultured epidermal cells. The inhibitory activity of pig- or human-derived EDF was reduced from 94% to 54% or from 56% to 32%, respectively, by heat treatment (60°C for 1 hr). Acid treatment (pH 3 for 3 hr) abolished the inhibitory activity. In contrast, other cell extracts (WI-38 human embryonic lung fibroblasts) or known growth factors such as EGF, PDGF, NGF, basic FGF, and TGF-β did not inhibit collagen contraction by fibroblasts, but actually increased it over the control values (Fig. 2*B*).

EGF, PDGF, basic FGF, and EDF were also compared for their ability to induce DNA synthesis in quiescent BALB/c 3T3 mouse fibroblasts. EGF, PDGF, and basic FGF all stimulated quiescent 3T3 cells in a dose-dependent fashion similar to that previously described (8–10). Half-maximal stimulation for EGF was obtained at 0.5 ng/ml, while EDF did not affect the [^{3}H]thymidine uptake by fibroblasts. However, in combination, EDF increased half-maximal stimulation of EGF 2-fold and the maximal stimulation of EGF at 10 ng/ml was increased 1.7-fold.

The dissimilar effects of EDF and other factors in the above assays provide supportive evidence that EDF is different from EGF, PDGF, basic FGF, TGF-β, and NGF.

Effects of EDF *in Vivo*. Surgically inflicted wounds in pigs (0.040 inch deep) covered by nonadhesive dressing soaked in PBS (control wounds) or in EDF at optimal concentration were used to evaluate the effects of EDF *in vivo*. The optimal concentration of EDF was established by preliminary *in vivo* titration on multiple properly spaced wounds (unpublished data). As documented in Fig. 3, EDF had a striking effect on the process of re-epithelization and the healing process in the dermis. Fig. 3*A* shows a section from the center of a 5-day-old control wound where there was virtually no evidence of epithelial activity. The growth of connective tissue, primarily of fibroblasts, was extensive, and there were also many inflammatory cells on the unhealed surface. The appearance of this section contrasts with that of an EDF-treated wound of the same age on the same pig (Fig. 3*D*). This section shows numerous branches of rapidly proliferating epidermal cells, with many mitotic figures, arising from a hair follicle. Under the crust of exudate the epidermal cells penetrated the new granulation tissue, which contained many inflammatory cells, and began to spread out in a thin layer (one to four cells thick) over its surface. Serial sections demonstrated that the new epidermal cells were of multiple origins, arising from several epithelial structures located in the undamaged deeper parts of the dermis. At this stage of healing the new granulation tissue in the treated areas was highly vascular.

In the control sections on day 12 (Fig. 3*B*) there was a multilayered loosely attached epidermis still lacking prominent rete ridges and the dermis was highly vascular and cellular. In EDF-treated wounds the epidermal cells spread rapidly over the surface of the exposed connective tissue and achieved complete coverage before day 8 (data not shown). By day 12 (Fig. 3*E*) there was a continuous, multicellular, hyperplastic layer of new epidermal cells. The rete pegs were shorter and their connections to the deep epidermal structures underwent atrophy. In EDF-treated wounds, compared to the controls, connective tissue proliferation appeared to be inhibited, cellularity and vascularity were decreased, and relatively thick new collagen matrix strands became apparent early.

By day 23 there was little difference between the biopsy specimens taken from the control and the experimental groups. However, the new epidermal layer was considerably thicker in the control than in the treated region, and some mitotic activity was still present in the basal keratinocytes of the control sections (compare Fig. 3 *C* and *F*). The granula-

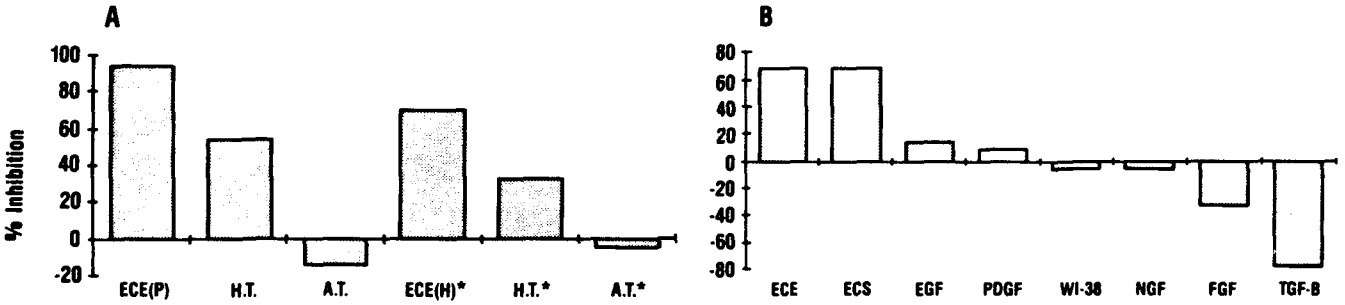

FIG. 2. Inhibitory activity of different growth factors on contraction of collagen sponges by fibroblasts. Calculations of percent inhibition of collagen contraction by different factors were based on the ratio of percent contraction of triplicate samples containing factor and control samples in the absence of factors. The SEM for percent contraction between triplicate samples varied from 0 to 4.9. (*A*) ECE(P), epidermal cell extract derived from pig keratinocytes grown *in vitro* (1:20 dilution); H.T., the same after heat treatment (60°C for 1 hr); A.T., after acid treatment exposure to pH 3 for 3 hr; *, the same as above, with cell extracts of human keratinocytes. (*B*) ECE, epidermal cell extracts (1:20 dilution); ECS, supernatant fluids from epidermal cell cultures (1:5 dilution); EGF, epidermal growth factor (200 ng/ml); PDGF, platelet-derived growth factor (100 ng/ml); WI-38, extracts of human embryonic lung fibroblasts (1:20 dilution); NGF, nerve growth factor (100 ng/ml); FGF, basic fibroblast growth factor (50 ng/ml); TGF-β, transforming growth factor β (1 ng/ml).

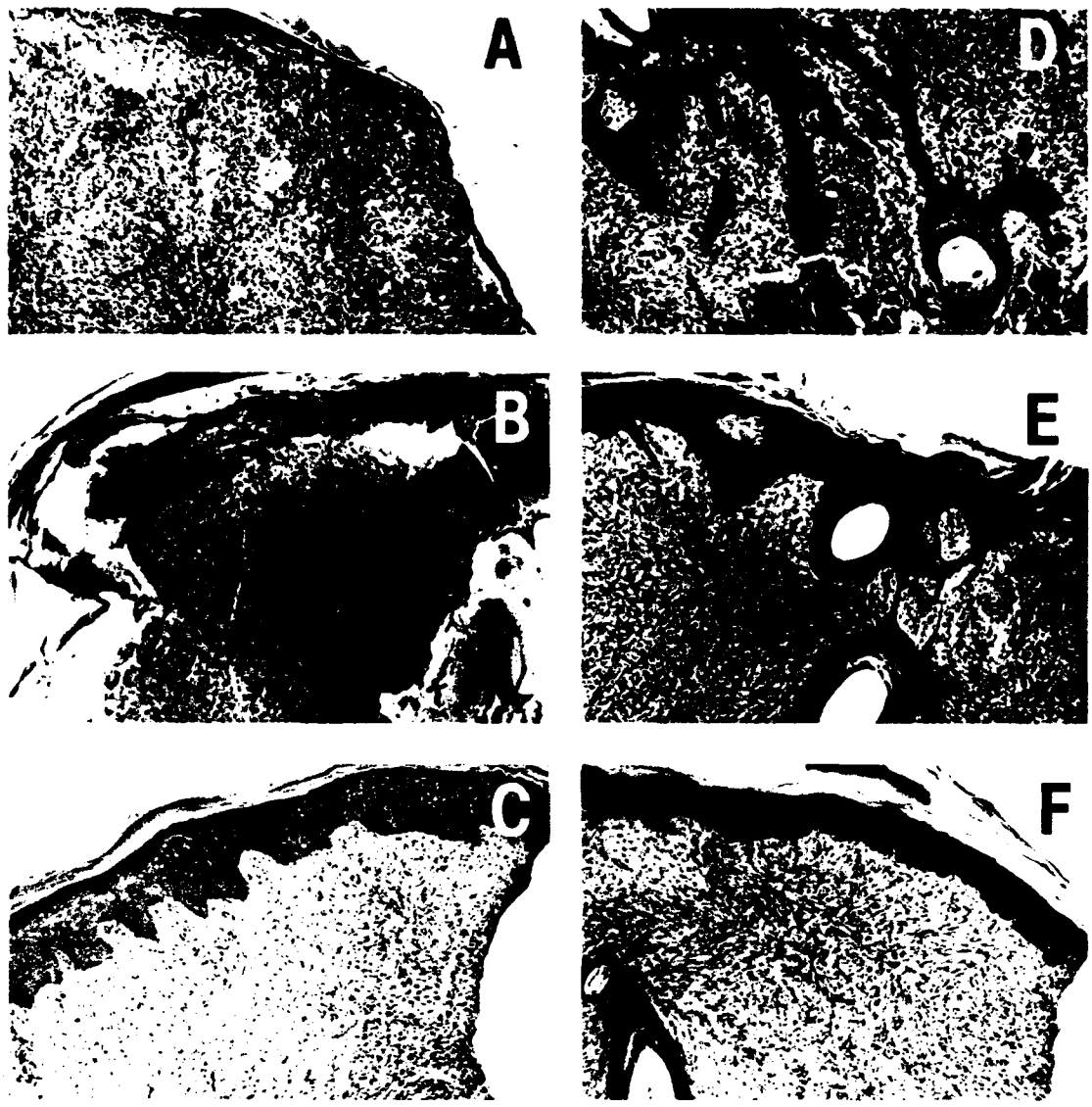

FIG. 3. Effects of epidermal cell extracts on wound healing. (×90.) (*A–C*) Histological sections of control wounds; (*D–F*) sections of epidermal cell extract-treated wounds (1:20 dilution). Days after wounding: *A* and *D*, 5; *B* and *E*, 12; *C* and *F*, 23.

tion tissue layer of the untreated control wounds was also considerably thicker and more cellular than in the treated areas.

Numerical Evaluation of the Effects of EDF on Healing Wounds. Table 1 summarizes the evaluation of histological specimens taken at 8 days after treatment of experimental wounds. It compares the effects of epidermal cell extracts (diluted 1:100) with those of EGF, urogastrone, TGF-α (all at 200 ng/ml), and controls treated with PBS. The density of fibroblasts was found to be lowest (0.11×10^6 per cm^2) in experimental wounds treated with EDF and resembled that of normal unwounded skin. Wounds treated with EGF and urogastrone showed the same fibroblast density as PBS-treated control (1.4×10^6 per cm^2). The highest numbers of fibroblasts were seen in wounds treated with TGF-α. In contrast, epidermal cells were most stimulated by EDF and EGF. EDF-treated wounds on day 8 were covered by seven layers of epidermal cells. Epidermal sprouts contained on average 80 layers of cells. EGF and urogastrone also stimulated re-epithelization, and TGF-α was the least effective stimulator of epidermal cell growth. However, compared to the control, all tested factors enhanced wound coverage by epidermal cells.

Table 1. Numerical evaluation of the effects of different growth factors on fibroblasts and epidermal cells as seen 8 days after wounding

	Fibroblasts			Epidermal cells	
Factor	Distance between nuclei, μm	Density, cells $\times 10^{-6}$/ cm^2	%	Top layer	Sprouts
EDF	30 ± 10	0.11	7.8	7	77
EGF	13 ± 4.3	0.6	42.8	1	30
Urogas.	9 ± 3	1.2	85.7	2	25
TGF-α	8 ± 2.6	1.6	114	0–4	0
PBS	8.5 ± 2.8	1.4	100	1–2	0
Normal skin	40 ± 13.3	0.06	4.3	4	0

Distance is mean ± SEM. Top layer is number of cell layers covering the surface of wounds. Sprouts refers to number of cell layers within the new granulation tissue arising from hair follicles.

DISCUSSION

The experiments described here reveal that the epidermal cells produce factors that have (*i*) a stimulatory effect on the growth of epidermal cells both *in vitro* and *in vivo* and (*ii*) an inhibitory effect on the contractility of fibroblasts *in vitro*

and on migration and/or proliferation of fibroblasts in healing wounds. This implies possible roles for locally produced factors in both positive- and negative-feedback mechanisms and the maintenance of homeostasis in the epidermis and dermis. Autocrine regulators previously identified in epidermal cells (chalones) have been found to be potent inhibitors of epidermal cell proliferation (11, 12). This led to the belief that epidermal cell growth is regulated by negative feedback. Factors that do stimulate epidermal cell growth—such as EGF, its human analog urogastrone, and closely related TGF-α—have all been isolated from tissues other than epidermal (13–15). The findings presented here also indicate that epidermal cells are capable of producing a factor that stimulates their own proliferation locally, and that its biological effect is different from that of EGF. EGF has been shown to enhance the general growth of epidermal cells *in vitro* (3). It was also shown recently that EGF and TGF-α can enhance the amount of TGF-α mRNA in cultured keratinocytes and its release into medium (16). As the medium used in our experiments did not contain EGF or TGF-α, the effects observed by us were most probably induced by a different factor produced by keratinocytes. In support of this view, our recent experiments indicate that the molecular weight of EDF is $\approx$1000 (unpublished results), clearly distinguishable from that of EGF or TGF-α. The application of EGF and TGF-α to experimental wounds was shown to accelerate the re-epithelization but also to promote connective tissue formation (17–19). Our experiments suggest that the stimulatory effect of EDF is more selective and may be confined to a specific subpopulation of epidermal cells, namely the stem cells. The appearance of colonies of epidermal cells after exposure to EDF indicated results resembling those recently described by Barrandon and Green (20). The limited number of colonies seen in our studies can be attributed both to the small number of stem cells in the epidermis and also to their possible loss in our separation procedure (5). Alternatively, the profound *in vivo* effect of EDF may be (*i*) potentiated by extracellular matrix materials or by other growth factors present in the wound, or (*ii*) mediated by another cell type. In addition to EGF and TGF-α, other well-characterized growth factors such as FGF, PDGF, and TGF-β were shown to stimulate proliferation of fibroblasts *in vitro* (9, 10) or *in vivo* (21). In contrast, EDF did not stimulate 3T3 fibroblasts and mildly suppressed proliferation of skin fibroblasts *in vitro*. Injuries that were treated with epidermal cell extract showed hypocellularity in newly formed granulation tissue. In comparison to wounds exposed to EGF, TGF-α, and urogastrone, fewer fibroblasts were observed in EDF-treated wounds.

The cells responsible for contracture are the fibroblasts, particularly the so-called myofibroblasts, characterized by myofibrils. Fibroblasts grown in tissue culture resemble myofibroblasts. Bell *et al.* (7) introduced a system for studying contracture of collagen gels by fibroblasts *in vitro* and have shown that the rate of collagen contracture is proportional to the density of fibroblasts in the collagen gel. In the results presented here, we have demonstrated that fibroblasts seeded into prefabricated collagen sponges can also induce contracture. This contracture can be completely inhibited by EDF but not by other known growth factors. EDF can therefore regulate contraction of collagen matrices either by controlling the numbers of fibroblasts or by influencing their state (replicating versus stationary). The important question to be answered is whether rapidly proliferating versus stationary fibroblasts produce different types of collagen and contribute to scar formation. It is well known that newly formed granulation tissue contains mainly type III collagen, characterized by thin filaments. Similarly, there is more type III and type V collagen in hypertrophic scars (22). In the wounds treated with EDF, bundles of relatively thick collagen strands were seen in the extracellular matrix as early as 5 days after wounding. The presence of bundles suggests that type I collagen might have been synthesized by resident fibroblasts, resulting in lesser contraction and no scarring.

The topic of investigation is whether factor(s) responsible for promoting re-epithelization and for suppressing fibroblasts are the same or different molecules. Experiments so far have shown that both properties are abolished by acid treatment but resist heat (60°C) and alkaline treatment. Another factor (TGF-β) has been shown to exhibit either growth-promoting or growth-suppressing effects (23–25) on different cell types. It appears therefore that epidermis may produce a factor responsible for both positive and negative regulatory effects in the skin. Should this prove to be a physiological phenomenon, elucidation of the factor's characteristics and mode of action would be of value both in understanding the normal mechanism of wound healing and, ultimately, in controlling its abnormal or clinically undesirable manifestations.

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